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Applicant FMC BIOPOLYMER AS et al	

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Norwegian Patent application no.

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Title : New mutant strains of *Pseudomonas fluorescens* and variants thereof, methods for their production, and uses thereof in alginate production

alginate lyase activity was present in the cultures and resulted in a low molecular weight, low viscosity polymer with rheology similar to printing grade alginate. The degradation by the lyase enzyme was remedied with the addition of proteolytic enzyme into the medium, Hacking A.J., et al., (1983) J. Gen. Microbiol., 129 , p. 3473-3480. After ten generations in continuous culture, non-mucoid variants appeared, Sengha S. S., et al., (1989) J. Gen. Microbiol., 135, p. 795-804. page 799, second paragraph.

An epimerase negative mutant of the opportunistic pathogen *P. aeruginosa* was reported by Chitnis et al. (1990) J. Bacteriol., 172, p. 2894-2900. Mucoid *P. aeruginosa* FRD1 was chemically mutagenized and mutants, which were incapable of incorporating guluronic acid (G)-residues into alginate were independently isolated. Assays using G-specific alginate lyase and ^{1}H-nuclear magnetic resonance analyses showed that G-residues were absent in the alginates secreted by these mutants. Goldberg and Ohman, 1987, J. Bacteriol., 169, p. 1593-1602, produced up to 1,7 g/l alginate from FRD1 in shake flasks. As usual for spontaneous alginate-producers non-mucoid revertants arise frequently (Flynn and Ohman, 1988, J. Bacteriol., 170, p. 1452-1460).

There is therefore still a need in the market for suitable sources for reliable alginate production in large amounts. In particular there is a need for stable sources producing large amounts of high quality alginate with defined structure and desired molecular weight, and especially for a source for the production of large amounts of biologically active alginate. Furthermore there is also a need for the production of pure mannuronan, which can be subjected to *in vitro* epimerization in order to obtain alginates with a predetermined guluronate residue (G)- content.

Summary of the invention

The present invention provides new mutant strains of *P. fluorescens*, which are stable and produce large amounts of alginate. Some embodiments of the invention is to provide variants thereof, which produce alginates with a defined structure with regard to content of mannuronate and guluronate residues, possible presence of, and determined level of O-acetyl groups and a desired molecular weight of the alginate molecules. Also high yielding mutants with regulated alginate production, is described. Other aspects of the invention are; methods of producing the novel mutant strains of *P. fluorescens* including variants thereof, and uses of the

resulting mutants in the production of alginates, in particular large scale production of alginates, and more particularly production of biologically active alginates, or pure mannuronan. The resulting alginates are applicable in different products such as nutrients and pharmaceuticals, they may also constitute an intermediate product suitable for further modifications by mannuronan–C5-epimerases, for instance the epimerases of US Patent No. 5, 939, 289.

Detailed description of the invention

The present invention provides a biologically pure bacterial culture of at least one mutant strain of *P. fluorescens* wherein said strain produces large amounts of alginate. In a preferred embodiment the said strain produces at least 10 g alginate per liter medium. Pure mutant strain of *P. fluorescens* bacterium and variants thereof, covered by the invention are exemplified by mutant strains selected from the group consisting of the mutant strains Pf201, Pf2012, Pf2013, Pf20118, Pf20137, Pf20118algIJΔ, Pf 20118algFΔ, Pf20118AlgLH203R and Pf201MC. In some embodiments, the invention relates to biologically pure bacterial culture of at least one strain of *P. fluorescens* wherein the strain produces alginate with alginate production characteristics of pf201 and variants thereof that retain such characteristics. Such "alginate production characteristics" may be one of more of the following: yield in terms of g/l and g/g carbon source, the average molecular mass, the degree acetylation and the G content of alginate produced.

In a second aspect the present invention comprises a pure mutant strain of *P. fluorescens* wherein the said mutant is capable of producing large amounts of an alginate consisting of mannuronate residues only. Preferred variants can be selected from the group consisting of the variant strains Pf2012, Pf2013, Pf20118, and Pf20137.

In a third aspect the present invention comprises a pure mutant strain of *P. fluorescens* wherein the said mutant is capable of producing large amounts of an alginate having a defined guluronate residue (G)-content between 0 and 30 %. Such embodiments may be produced by exchanging the wild type *algG* with a mutant gene encoding a mannuronan C-5-epimerase enzyme with lower specific activity than the wild type enzyme.

In a fourth aspect of the invention the pure mutant strain of *P. fluorescens* is capable of producing large amounts of an alginate without, or with a reduced number

of O-acetyl groups. Such embodiments may be produced by deleting parts of, or all of the genes *algI*, *algJ*, and/or *algF*. The mutant variant strains Pf20118algIJΔ and Pf20118algFΔ are capable of producing large amounts of an alginate without, or with a reduced number of O-acetyl groups, and represents preferred embodiments of this aspect of the invention.

In a fifth aspect of the present invention the pure mutant strain of *P. fluorescens* is capable of producing large amounts of an alginate with a desired molecular weight%. Such embodiments may be produced by exchanging the wild type *algL* with a mutant gene encoding an alginate lyase enzyme with lower specific activity than the wild type lyase enzyme. The pure mutant variant strain Pf20118AlgLH203R represents a preferred embodiment of the said mutant, which is capable of producing large amounts of an alginate with a desired high molecular weight.

In a sixth aspect of the present invention the pure mutant strain of *P. fluorescens* capable of producing large amounts of alginate, comprises an alginate biosynthetic operon regulated by an inducible promoter different from the naturally occurring promoter, and optionally one or more effector genes. The inducible promoter is preferably a *Pm* promoter, and the effector gene is *xylS*. According to one preferred embodiment the said mutant strain is Pf201MC.

A seventh aspect of the invention provides a method of producing the novel mutant strain of *P. fluorescens* of the invention, wherein :

(a) a wild-type strain of *P. fluorescens* is contacted with a mutagenic agent, and

(b) the treated bacteria of step (a) are grown in the presence of one or more antibiotics , and

(c) antibiotic resistant mucoid mutants are isolated by selection, and

(d) the alginate production properties of the isolated mucoid mutants of step (c) are determined.

The mutagenic agent of step (a) in the method is preferably nitrosoguanidine, and the antibiotics applied in step (b) is a β-lactam and/or aminoglycoside antibiotic, preferably the antibiotic is carbenicillin. The antibiotic may be present in the range of 800-1000 μg/ml medium, and more preferably in amounts of 900 μg/ml medium.

In still another aspect the present invention provides a method of producing a mutant strain of *P. fluorescens* capable of producing large amounts of alginate where

the alginate biosynthetic operon is regulated by an inducible promoter different from the naturally occurring promoter, and optionally one or more effector genes, wherein:
(i) the alginate biosynthetic operon promoter of a wild type strain of *P. fluorescens* is exchanged by an inducible promoter by homologous recombination, and
(ii) optional effector genes are introduced into the bacterium of (i) by homologous recombination, transposon mutagenesis or by means of a plasmid, and
(iii) mutants are grown and then isolated by selection, and
(iv) the alginate production properties of the isolated mutants of (iii) are determined.
In one embodiment of the method according to the invention the inducible promoter is *Pm* from *P. putida* Tol-plasmid.

In other aspects the invention provides use of biologically pure bacterial culture of at least one mutant strain of *P. fluorescens* as described herein for the production of alginate, and use of the alginate produced in the preparation of a pharmaceutical or nutrient product, or as an intermediate product for *in vitro* C-5-epimerization.

The mutant strains; Pf201, Pf2012, Pf2013, Pf20118, Pf 20137, Pf20118algFΔ, Pf20118algIJΔ, Pf20118AlgLH203R, and Pf201MC of the invention have been deposited in The National Collections of Industrial Food and Marine Bacteria Ltd. (NCIMB) the 16th of July, 2002 under the following accession numbers ; 41137, 41138, 41139, 41140, 41141, 41142, 41143, 41144 and 41145 repectively.

Definitions

The novel mutant strains and variants thereof of the present invention, produce alginate in large amounts, with "large amounts" as used herein, are meant at least 10 g alginate per liter. Amounts of 10 g alginate per liter medium can be achieved from 40 g carbon source per liter medium, suitable carbon sources are fructose, glucose, glycerol, sucrose, lactate (lactose) or galactose, but also other C-sources might be used. Preferably 40 g fructose per liter medium is used as carbon source for the production of alginate.

The mutant strains and the variants thereof, according to the present invention, are "stable", that is, they do not revert to strains, which do not produce alginate, when they are grown over 60 generations.

The "mutant strain" used herein comprises mutant strains of *P. fluorescens* Pf201, as well as variant mutant strains, which all produce alginate in large amounts.

In preferred embodiments, "mutant strain" refers to mutant strains of *P. fluorescens* Pf201 which all produce alginate in large amounts. The variants might be a result of further mutagenesis of the Pf201 mutant strain and/or further genetic engineering, or a result of genetic engineering of a wild type *P. fluorescens* strain. The variants will produce large amounts of alginates of certain defined structures. Also variants containing any combination of the herein defined mutations are considered covered by this expression.

The alginate produced according to the invention will have a "desired molecular weight". Preferably alginate with molecular weight (Mw.) in the range from 100 000 to 3 000 000 Dalton, more preferable within 200 000 to 2 000 000 Dalton, and most preferably above 300 000 Dalton, is produced.

With the expression "biologically active alginate" used herein, is meant an alginate having an impact on a biological system, i.e. certain bioactive alginate molecular structures are known to induce biological responses in certain cellular systems. Such biological alginates have a lower content of guluronic acid (guluronate) residues, from 0 to 30 % of the total uronic acid content, and preferably the guluronic acid residue content is between 1 and 10 %.

Description of figures

Figure 1: Restriction endonuclease maps of the suicide vectors pHE55 and pMG48, confer Table 1. Only unique restriction enzyme sites shown.

Figure 2: Growth and alginate production in fermentations with mutant strains of *P. fluorescens* NCIMB 10525.

Figure 3: ^{1}H-NMR-spectra of alginate produced by *P. fluorescens* mutant strains Pf201 and Pf20118. The ^{1}H-NMR-spectra of mannuronan from the other epimerase negative mutants (Table 3) were identical with the one for Pf20118.

Figure 4: The alginate biosynthetic operon and the upstream open reading frame from *P. fluorescens* are shown. The cloned fragments are marked as boxes on the map line. Only restriction sites used for cloning are shown. The total length is 18 kb.

Figure 5: Restriction endonuclease map of the plasmid pMC1. Only unique restriction enzymes are shown.

General description of Materials and Methods

Starting materials and culture media used for growth of bacteria

The bacterial strains, phages and plasmids used in the present invention are listed in Table 1 below. *E. coli* and *P. fluorescens* strains were routinely grown in LB medium (10 g/l tryptone, 5 g/l yeast extract, and 5 g/l NaCl) or on a LA-medium, which is LB-medium containing 15 g/l agar, at 37°C and 30°C, respectively. Pseudomonas Isolation agar (PIA, Difco) was also used for propagation of *P. fluorescens*. *E. coli* used for λ phage propagation was grown in LB-medium supplemented with maltose (0,2%) and $MgSO_4$ (10 mM). Antibiotics, when used in routine growth experiments, were present at the following concentrations: Ampicillin 100-200 µg/ml, kanamycin 40 µg/ml, tetracycline 12,5 µg/ml (*E. coli*) and 30 µg/ml (*P. fluorescens*).

Production of *P. fluorescens* alginate; culture media and growth conditions

Culture media:

Production of alginate in shake flask experiments was performed in PIA medium containing bacteriological peptone (20 g/l), $MgCl_2$ (1,4 g/l), NaCl (5 g/l), K_2SO_4 (10 g/l) and 87% glycerol (20 ml/l) or in PIA-medium with reduced salt (PIA-medium without K_2SO_4). The proteases (Alkalase 2,4L (0,15 ml/l) and Neutrase 0,5L (0,15 ml/l)) were added to reduce extracellular alginate lyase activity, unless otherwise stated. Alkalase and Neutrase were purchased from Novo Nordisk.

Production of alginate in fermentor was performed in PM5-medium containing: fructose (40g/l), yeast extract (12 g/l), $(NH_4)_2SO_4$ (0.6 g/l), $Na_2HPO_4x2H_2O$ (2 g/l), NaCl (11.7 g/l), $MgSO_4 x\ 7H_2O$ (0.3 g/l) and clerol FBA622 (antifoam) (0,5 g/l). The proteases (Alkalase 2,4L (0,25 ml/l) and Neutrase 0,5L (0,25 ml/l) were added to reduce extracellular alginate lyase activity.

Preparation of standard inoculum (frozen culture with glycerol as cryoprotectant)

A colony from agar plate (incubated at 30 °C for 2-3 days, PIA-medium) is transferred to a shake flask (500 ml, baffled) with 100 ml LB-medium. The shake flask is incubated at 30°C for 16 - 20 hours in an orbital shaker (200 rpm, amplitude 2,5 cm). For preservation sterile glycerol is added to the broth to a concentration of 15%. The mixture is transferred to sterile cryo vials (Nunc) and stored at –80 °C.

Preparation of inoculum for production experiments in shake flasks and fermentor

1 ml standard inoculum is transferred to a shake flask (500 ml, baffled) with 100 ml LB-medium. The shake flask is incubated at 30°C for 16 - 20 hours in an orbital shaker (200 rpm, amplitude 2,5 cm).

Alginate production in shake flask

1-2 vol-% inoculum (see above) is transferred to a shake flask (500 ml, baffled) with 100 ml PIA-medium or PIA-medium with reduced salt. The shake flask is incubated at 25°C for 48 hours in an orbital shaker (200 rpm, amplitude 2,5 cm).

Alginate production in fermentor

2-3 vol-% inoculum from shake flask is transferred to a 3-litre fermentor (Applicon), with 1,4 litre PM5-medium. The fermentations are performed at 25 °C. pH from start is adjusted to 7.0-7.2. pH is controlled at 7.0 with NaOH (2 M) and the pH-control is activated when the pH reaches this value. The airflow trough the culture medium is 0,25 litre / litre medium (vvm) for the first 8-10 hours, thereafter it is increased in steps up to 0,9-1,0 vvm. The dissolved oxygen is controlled at 20 % of saturation by automatic control of the stirrer speed.

Applied standard techniques

Plasmid isolation, enzymatic manipulations of DNA and gel electrophoresis were performed by the methods of Sambrook and Russell, 2000, Molecular Cloning: A Laboratory Manual (Third Edition). Cold Spring Harbor Laboratory Press. Qiaquick Gel Extraction Kit and Qiaquick PCR purification kit (Qiagen) was used for DNA-purifications from agarose gels and enzymatic reactions, respectively. Transformation of *E. coli* was performed as described by Chung et al., 1989, Proc Natl Acad Sci USA, 86, p. 2172-2175 or by use of heat-shock-competent rubidium chloride cells. PCR for cloning and allele identification was performed using the Expand High Fidelity PCR-system (Boehringer Mannheim). As templates were used either plasmid DNA or 1 μl of an over-night *P. fluorescens* culture. In the first denaturation step the reaction mixtures were heated to 96 °C for three minutes to ensure both cell lysis and full denaturation of the DNA. Site-specific mutagenesis was performed using QuickChange Site-Directed Mutagenesis Kit (Stratagene). Primers given in Table 2 were purchased from Medprobe or from MWG-Biotech AG. Nucleotides in the

primers, which are different from those of the wild-type sequence are written in bold, and restriction-enzyme sites are underlined. DNA sequencing was performed using a Big-Dye kit (Applied Biosystems).

Construction of suicide vectors for use in *P. fluorescens*

In order to achieve homologous recombination in *P. fluorescens* two different suicide vectors, pHE55 and pMG48, were constructed, confer Figure 1. The construction of pHE55 is described in Table 1. It is an RK2-based vector lacking the gene encoding TrfA, which is necessary for replication of the plasmid. It further confers resistance to ampicillin and tetracycline, which can be used for selecting integrants. Expression of *sacB* encoding levan sucrase from *Bacillus subtilis* has been shown to be lethal for many gram-negative bacteria when grown on 5 % sucrose (Gay et al.,1985, J. Bacteriol., 164, p. 918-921). In strain NCIMB 10525 of *P. fluorescens*, however, growing non-mucoid and tetracycline resistant transconjugants on sucrose resulted in glassy colonies, as if the strain uses the sucrose to produce a polymer. SacB and sucrose selection could then not be used for this strain to positively select double cross-overs. pHE55 was used as a suicide vector in some experiments, where alginate production could be used as a marker.

The plasmid pMG48 was constructed as an alternative recombination vector. The *sacB*-gene of pHE55 was replaced by a gene encoding a TrfA-LacZ-fusion protein, as described in Table 1. This protein shows β-galactosidase activity, but the essential parts of TrfA is missing. Using plates containing XGal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside), 60 μl of a 20 mg/ml stock solution was added to each agar plate used for screening. The β-galactosidase activity allow for blue/white screening both for integrants (blue colonies) and later for the second recombination event (white colonies).

Homologous recombination

The DNA sequence containing the mutation of interest, either a point-mutation, insertion or deletion together with flanking DNA of at least 0.5 kb on each side was cloned into a suicide vector, either pHE55 or pMG48. *E. coli* S17.1 transformed with the plasmid of interest and the *P. fluorescens* strain to be mutated were incubated in LB-medium over-night. They were then incubated in fresh LB-medium, 1 % inoculum was used. *E.coli* was grown for two hours, *P.fluorescens* for four hours prior to

conjugation. One ml of each culture were then mixed and centrifuged for 15 min. at 3000 rpm. Most of the supernatant was removed, and the cells were resuspended in the remaining liquid. The droplet containing the cells was transferred to LA-medium, and incubated at 30 °C over-night. The cells were removed by a sterile spatula, resuspended in LB-medium, and dilutions were plated on *Pseudomonas* Isolation agar (PIA, Difco) with appropriate antibiotics and X-Gal when the vector allowed for blue/white selection. A non-mucoid transconjugant colony of each mannuronan-producing strain was incubated in 2-6 sequential liquid over-night cultures in the absence of tetracycline to allow loss of the integrated plasmid. Exponentially growing cultures were diluted 10^4-10^9 fold and plated on the appropriate medium to screen for the different strains.

Measurement of G-content and degree of O-acetylation of the alginate by NMR-spectroscopy.

Samples from fermentations were diluted in 0.2 M NaCl and centrifuged to remove the bacterial cells. For preparation of samples for determination of degree of acetylation, alginate was precipitated from the cell free supernatant by addition of one volume isopropanol (4°C), and thereafter collected by centrifugation. The precipitated alginate was then washed twice with 70% ethanol, once in 96 % ethanol, and redissolved in distilled water before further treatment. For preparation of samples for determination of G-content the alginate in the cell free supernatant was deacetylated by mild alkaline treatment as described in Ertesvåg and Skjåk-Bræk, 1999, In Methods in biotechnology 10, Carbohydrate Biotechnology Protocols. Bucke, pp 71-78. Humana Press Inc. Deacetylated alginate was isolated from the cell free supernatant by acid precipitation by adding HCl to pH 2. The precipitated alginate was collected by centrifugation, redissolved in distilled water and neutralized by alkali. To reduce the viscosity of the polymer for NMR analysis the samples were degraded by mild acid hydrolysis to a final average degree of polymerisation (DPn) of about 35, that is 35 residues in the polymer chain, neutralized and freeze dried, Ertesvåg and Skjåk-Bræk, 1999, *supra*. NMR-spectra were obtained using a Bruker 300-MHz Spectrometer. The spectra were integrated, and the fractions of guluronate residues (F_G), mannuronate block residues (F_{MM}) and alternating block residues ($F_{MG=GM}$) and degree of acetylation were calculated as described in Grasdalen, 1983,

Carbohydr. Res., 118, p. 255-260 and Skjåk-Bræk, Grasdalen and Larsen, 1986, Carbohydr. Res., 154, p. 239-250.

Measurement of the intrinsic viscosity of the alginate and direct measurement of alginate content in fermentation samples.

The alginate produced was isolated, deacetylated, acid precipitated, redissolved and neutralized as described above. The neutralized alginate solution was added isopropanol to precipitate the alginate a second time. The precipitated alginate was washed twice with ethanol (first 70% and then 96% ethanol), redissolved in distilled water and dialyzed against distilled water for 48 hours . After dialysis the sample was freeze dried and weighed. The intrinsic viscosity of the alginates was determined on a Scott-Geräte apparatus with automatic dilution, using an Ubbelodhe capillary (Φ=0,53 mm) at 20°C and an added salt concentration of 0,1 M NaCl. The principle of the method is as described in Haug and Smidsrød, 1962, Acta. Chem. Scand., 16, p1569-1578.

Enzymatic determination of alginate content in fermentation samples.

Alginate content was measured using the M-specific lyase from abalone and G-lyase from *Klebsiella aerogenes* as described by Østgaard, 1992, 19, Carbohydr. Polymers, p. 51-59.

Samples from fermentations were diluted (2-20 times) in 0,2 M NaCl, centrifuged to remove bacterial cells, and deacetylated, as described above. The deacetylated samples were then diluted in buffer (Tris-HCl (50 mM), NaCl (0,25M), pH 7,5) to a final concentration of 0,005 – 0,05 % alginate. LF 10/60 (FMC Biopolymer AS) or mannuronan, produced and measured as described herein, were used as alginate standards in the assay. For the assay one volume of sample, or standard and 0,06 volumes of alginate lyase solution (about 1 u/ml) are added to two volumes of buffer (Tris-HCl (50 mM), NaCl (0,25M), pH 7,5) and incubated for 3 hours at 25°C. The absorbance at 230 nm is recorded before and after the incubation. The differences in the A230 nm values before and after the incubation are used for calculation of the alginate content in the sample. The results, using this assay, correlate very well with the direct measurement of alginate content described above.

Determination of lyase activity

Bacterial cells from fermentations were collected by centrifugation, resuspended in buffer (Tris-HCl (50 mM), NaCl (0,25M), pH 7,5) to an optical density of 3-10 at 660 nm and sonicated. The extracts after sonication were investigated for lyase activity. M-specific lyase from abalone (described by Østgaard, 1992, 19, Carbohydr. Polymers, p. 51-59) was used as standard. The lyase activities in samples were determined by measuring the degradation rate of mannuronan using a Scott-Geräte Ubbelodhe (instrument nr. 53620/II). Mannuronan (1 mg/ml) was dissolved in buffer (Tris-HCl (12,5 mM), NaCl (62,5 mM), pH 7,5). 4 ml of mannuronan substrate solution and 0,4 ml of diluted standard solution, or sample were added to the Ubbelodhe capillary. The time for the solution to pass the capillary of the Ubbelodhe was measured every 2 minute over a time period of one hour. The analysis was performed at 25°C. Based on the data from the analyses, the degradation rate of mannuronan was calculated and correlated to the lyase activity in the sample. A standard curve was obtained using the abalone M-lyase as a standard (0.005 – 0.05 u/ml). 1 unit of lyase activity is defined as described by Ertesvåg et al., J. Bacteriol. (1998), 180, p. 3779-3784.

Table 1. Bacterial strains, plasmids and phages used

Strains	Description	Reference
E. coli S17.1	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*Δ (*lac-proAB*), contains the necessary genes for replication and transfer of RK2.	Simon et al, 1983, Biotechnol. 1, p. 784-791.
E. coli SURE	e14– (*McrA*–) D(*mcrCB-hsdSMR-mrr*)171 *endA*1 *supE*44 *thi-1* *gyrA*96 relA1 *lac recB recJ sbcC umuC*::Tn5 (*Kanr*) *uvrC* [F' *proAB lacIqZD*(M15 Tn10 (*Tetr*)]	Stratagene
E. coli XL1-Blue MRA	Δ(*mcrA*) 183 Δ(*mcrCB-hsdSMR-mrr*) 173 *endA*1 *supE*44 *thi-1* *gyrA*96 *relA1 lac*	Stratagene
E. coli XL1-Blue MRA(P2)	XL1-Blue MRA (P2 lysogen)	Stratagene
P. fluorescens NCIMB10525	Non mucoid *P. fluorescens* wild type	NCIMB
Pf201	*algG*$^{+}$, mucoid *P. fluorescens*	This work
Pf2012	mannuronan-producing mutant, *algG* *D*361N	This work
Pf2013	mannuronan-producing mutant, *algG* G430D	This work
Pf20118	mannuronan-producing mutant, *algG* R408L	This work
Pf20137	mannuronan-producing mutant, *algG* S337F	This work
Pf20118algFΔ	*algF* in-frame deletion mutant of Pf20118.	This work
Pf20118algIJΔ	*algIF* in-frame deletion mutant of Pf20118.	This work
Pf20118algLΔ	*algL* in-frame deletion mutant of Pf20118	This work
Pf20118algLH 203R	Pf20118-derivate encoding the AlgLH203R mutant protein	This work
Pf201Δ*algG*	*algG* in-frame deletion mutant	This work

Pf201MC	Derivative of Pf201 in which the alginate biosynthesis is controlled by the inducible promoter Pm.	This work
Phages		
λDashII	λ cloning vector	Stratagene
Pfλ1	λ DashII in which an 15 kb insert of *Sau*AI-partially digested genomic DNA from *P. fluorescens* NCIMB10525 containing *alg'EGXLIJFA* has been inserted.	This work
Plasmid		
pCVD442	Ori R6K, Ap^r	Donnenberg and Kaper, 1991, 59, p. 4310-4317.
pJB3Tc20	RK2-based vector, Ap^r, Tc^r	Blatny *et al.*, 1997, Appl. Environ. Microbiol., 63, p. 370-379.
PJB3Tc20trfA	Derivative of pJB3Tc20 from which a 1.0 kb *Bsa*AI-*Nde*I- DNA-fragment encoding TrfA was deleted.	This work
pHE55	Derivative of pJB3Tc20trfA in which a 2.6 kb *Pst*I-*Xba*I- DNA-fragment from pCVD442 encoding SacB from *Bacillus subtilis* was inserted.	This work
pJB1002	RK2-based vector encoding a TrfA-LacZ-fusion protein	Karunakaran *et al.*, 1998, J. Bacteriol, 180, p. 3793-3798.
pGEM5	ColE1. Ap^R.	Promega
pMG47	Derivative of pHE55 in which a 4.1 kb *Nhe*I-*Pst*I-DNA-fragment from pJB1002 encoding a TrfA-LacZ-fusion protein replaced a 2.6 kb *Xba*I-*Pst*I DNA-fragment encoding SacB:	This work
pMG48	Derivative pMG47 in which a 0,36 kb *Sph*I-*Sap*I DNA-fragment containing the polylinker of pGEM5 has been inserted.	This work
pBBg10	9.9 kb *Bgl*II-*Bam*HI insert from the *Pseudomonas aeuginosa* alginate biosynthetic operon containing alg'KEGXLIJF. Ap^r.	Gift from A. Chakrabarty.
pGEM11	ColE1. Ap^R.	Promega
pMG24	pGEM11 containing a 1 kb *Sal*I-DNA-fragment from Pfλ1 encoding part of *algE*.	This work
pMG25	pGEM11 containing a 4,2 kb *Sal*I DNA-fragment from Pfλ1 encoding sequences downstream of the alginate operon.	This work
pMG26	pGEM11 containing a 4.6 kb *Sal*I-DNA-fragment from Pfλ1 encoding *algGXLI'*.	This work
pMG27	pGEM11 containing a 4.8 kb *Sal*I-DNA-fragment from Pfλ1 encoding *alg'IJFA*.	This work
pLitmus28	ColE1. Ap^R.	New England Biolabs
pMG23	pLitmus28 in which a 1.8 kb PCR amplified *Bgl*II-*Pst*1 DNA-fragment containing *algG* and 135 bp of *algX* was inserted. The primers PfalgG3r and PfalgG4f were used.	This work
pMG31	Derivative of pHE55 in which an 1.8 kb *Bgl*II-*Xba*I-DNA- fragment encoding AlgG from pMG23 was inserted.	This work
pMG49	pMG27-derivate from which a 1.4 kb *Nru*I-*Hpa*I DNA-fragment was deleted, creating an in frame *algI"J*-deletion	This work
pMG50	pHE55 with 3441bp *Sac*I-*Xba*I insert from pMG49 containing *algIJ*Δ.	This work
pMG77	Derivative of pMG27 where a *Sac*II-site was introduced using the primerpair algF-*Sac*II-1 and algF-*Sac*II-2 (table 2).	This work

pMG78	Derivative of pMG77 from which a 285 bp *Sac*II-DNA fragment in *algF* was deleted.	This work
pMG79	Derivative of *Sph*I-*Spe*I-restricted pMG48 in which a 1.7 kb *Nsp*I-*Nhe*I-DNA fragment from pMG78 was inserted.	This work
pMG67	Derivative of pMG26 in which an *Age*I-site and an algLH203R was introduced using the primers AlgLH203R1 and AlgLH203R2.	This work
pMG70	Derivative of pMG48 into which a 2.5 kb *Pst*I-*Not*I-DNA-fragement from pMG67 was inserted into the *Nsi*I and *Not*I-sites of the vector	This work
pJB658 celB	Expression vector containing the Pm-promoter and *xylS*. Ap^r.	Blatny *et al.*, 1997, Plasmid, 38, p. 35-51.
pHE138	Derivative of pJB658celB in which a 0.8 kb *Nde*I-*Nsi*I-digested PCR-fragment encoding the N-terminal part of AlgD was inserted into the *Nde*I and *Pst*I-sites replacing *celB*.	This work
pHE139	Derivative of pMG48 in which a 0.7 kb *Bsp*LUIII-*Spe*I-digested PCR-fragment encoding the C-terminal part of the ORF upstream of the alginate promoter was inserted into the *Nco*I and *Spe*I-sites.	This work
pHE140	Derivative of pHE138 from which a 0.6 kb *Nsi*I-DNA-fragment had been removed, and the protruding ends removed by T4-DNA-polymerase.	This work
pHE141	A *Bgl*II-linker was inserted into *Nsi*I-digested pHE139 which had been made blunt using T4-DNA-polymerase.	This work
pHE142	A *Not*I-linker was inserted downstream of *xyl*S in pHE140 partially digested with *Eco*57I.	This work
pMC1	A 2.3 kb *Not*I-*Bam*HI-DNA-fragment from pHE142 was inserted into *Not*I-*Bgl*II-digested pHE141.	This work

Table 2: Primers used

Name	Sequence*
PfalgG3r	CAGGCTGCAGCACGGTTCGGC
PfalgG4f	**AAAAAGATCTA**GTCGACTCGTACATGCACC
PfacetylFw	CTGCTGGTGGTGATGGGCTGGG
PfacetylRev	AGACGCGCACGAAGCTTGAGCC
algF-*Sac*II-1	GTCAAACTCGCCGC**G**GATCACTAC
algF-*Sac*II-2	GTAGTGATC**C**GCGGCGAGTTTGAC
algF-1-Fw	AGCGATGACTTCAAGAACAACCCG
algF-2-Rev	CAATTTGGGTCAGAGCTACGAAGG
algLH203R1	AACCAACAACC**GG**TCCTACTGGGCCGCC3'
algLH203R2	GGCGGCCCAGTAGGA**CC**GGTTGTTGGTT
PfalgL-BspHI-pMG26	**AAAAAAA**G**TC** ATGAGGTTACCTATGCAGAAGTTATTG
PfalgLRev1	AAAGATCGGCAAGAACAGAAACAGG
HypBspLUIII	**GTTACATGT**CAGCCGCAATACCTCGACC
HypSpe	**GTTACTAGT**TTATTCGGGGGCGTGATCG
AlgDNdeI	GGTAATT**CAT**ATGCGCATCAGCATATTTG
AlgDNsiI	**GTAAT**GCATGTAGTACTGGGACAGG

* The primers are written in the 5'-3' direction. Nucleotides not found in the original sequence are shown in bold. Introduced restriction-sites are underlined.

Examples

Example 1

Preparation of mutant strain Pf201

The wild type *P. fluorescens* NCIMB10525 was purchased from The National Collections of Industrial Food and Marine Bacteria Ltd. (NCIMB). The wild type does not produce significant amounts of alginate. In order to isolate alginate over-producing mutants exponentially growing cells of *P. fluorescens* NCIMB 10525 were subjected to nitrosoguanidine (NG) mutagenesis. The strain was grown in nutrient broth (CM67, Oxoid) with 0.5% yeast extract and washed twice in 0.1M citrate buffer (pH 5.5) before treating the cells with 25 ug/ml nitrosoguanidine (NG) in citrate buffer for 1 hour at 30°C . The mutagenized cells were washed with 0,1 M phosphate buffer pH 7,0 containing KH_2PO_4 (13,6 g/L) and NaOH (~2,32 g/L) and inoculated (2 %) into nutrient broth with yeast extract. The cells grew overnight and were then frozen as 1 ml aliquots of NG stock.

Dilutions of the culture were plated on PIA-medium containing carbenicillin (900 µg/ml) and incubated at 30°C. A few mucoid mutants were observed. From the screening, which included inspection of more than $4*10^5$ colonies, the two most mucoid mutants were selected for further evaluation in fermentor studies. The better mutant, Pf201 yields in fermentation 11-13 g alginate per liter PM5-medium containing 40 g fructose as carbon source per liter, as depicted in Figure 2. For growth conditions and medium composition, it is referred to Materials and Methods. The alginate produced by the Pf201 mutant using the PM5-medium containing fructose, and under standard growth conditions, contains about 30% G (guluronate residues) with complete absence of G-blocks as can be estimated from Figure 3. Based on the unique alginate production properties, the Pf201 strain was selected for further strain development. The *P. fluorescens* mutant Pf201 of example 1 is deposited in NCIMB under the accession number 41137.

Example 2

Cloning and sequencing of parts of the alginate biosynthetic operon

A gene library of the wild-type strain NCIMB 10525 was constructed in λDASH II (lambda Dash II) (purchased from Stratagene). Chromosomal DNA was isolated as described by Ausubel et al., 1993, Current protocols in molecular biology. Greene Publishing Associates, Inc and John Wiley & Sons Inc, New York. The gene-library

was then constructed by inserting partially *Sau*3AI-digested chromosomal DNA from NCIMB 10525 into *Bam*HI-digested lambda Dash II and infecting *E. coli* XL1-Blue MRA(P2) with the *in vitro*-packaged phages according to the manufacturers instructions (Stratagene *Bam*HI/Gigapack III Gold Extract). Labelling of DNA-probe and detection of hybridizing λ-clones were done by use of DIG DNA Labelling and Detection Kit (Boehringer Mannheim) according to the manufacturers instructions. A 3.8 *Mfe*I-*Nco*I DNA-fragment from pBBg10 containing *alg*G flanked by parts of *alg*E and *alg*X from *P. aeruginosa* was labelled and used to screen the *P. fluorescens* library. One hybridizing phage, designated Pfλ1, was detected using this system and λ DNA was isolated using Lambda Midi Kit (QIAGEN). The insert was subcloned as *Sal*I-digested DNA-fragments into pGEM11 resulting in the four subclones pMG24-27. Sequencing of the ends of the subclones and comparison with the alginate biosynthetic operon of *P. aeruginosa* revealed that Pfλ1 contains the downstream part of the alginate biosynthetic operon from the 3' part of *algE* (Figure 4).

pMG26 and pMG27 were sequenced by Quiagen Sequencing & Genomics to obtain the full sequence of *algGXLIJFA*. This gene organization seems to be similar to previously reported alginate biosynthetic clusters in; May and Chakrabarty, 1994, Trends Microbiol., 2, p. 151-157 , Rehm et al., 1996, J. Bacteriol., 178, p. 5884-5889, Penaloza-Vazquez et al., 1997, J Bacteriol., 179, p. 4464-4472, Vazquez et al., 1999, Gene, 232, p. 217-222. The sequence has been submitted to GenBank and given the accession number AF527790.

Example 3

Preparation of epimerase negative variant strains

The mutant strain Pf201 of Example 1 was subjected to further mutagenesis using nitrosoguanidine using a modification of the method described in Example 1. Exponentially growing cells of *P. fluorescens* NCIMB 10525 were subjected to nitrosoguanidine (NG) mutagenesis: The bacterial cells were washed twice with equal volume of Tris/maleic acid (TM) buffer pH 6,0 containing NH_4SO_4 (1,0 g/L), $CaCl_2*2H_2O$ (4,4 mg/L), KNO_3 (6,1 mg/L), maleic acid (5,8 g/L), Tris (hydroxy methyl)-amino methane (6,05 g/L), $FeSO_4*7H_2O$ (0,25 mg/L) and $MgSO_4*7H_2O$ (0,1 g/L). Cells were re suspended in 80% of original culture volume of TM-buffer and exposed to NG (50 µg/ml) for 1 hour at 30°C. The mutagenized cells were washed

with 0,1 M phosphate buffer pH 7,0 containing KH_2PO_4 (13,6 g/L) and NaOH (~2,32 g/L) and inoculated (2 % inoculum) into LB-medium and incubated over-night. The death rate of the mutagenesis procedure was calculated to approximately 90 % using a non-mutagenized aliquot of the culture as control. After mutagenesis the culture was grown in LB-medium over-night, and dilutions of the cells plated on LA-medium containing G-lyase from *Klebsiella aerogenes* (about 0.1 u/dish), as described by Chitnis. et al, 1990, J. Bacteriol., 172, p. 2894-2900. This G-lyase cleaves only the G-M (guluronate-mannuronate residue) and the G-G (guluronate-guluronate residue) bonds in alginate, Haugen et al, 1990, Carbohydr. Res., 198, p. 101-109.

Mucoid mutants appeared at a frequency of about 1 in 7500 on such selective plates. One mucoid mutant was isolated and designated Pf20118. Pf20118 was grown in a fermentor under standard growth conditions using PM5 medium, confer Materials and Methods. The polymer produced was analyzed by ^{1}H-NMR spectroscopy. The results of this analysis showed that the mutant produced pure mannuronan, confer Figure 3. Several fermentations were performed with Pf20118 using standard growth conditions and the PM5-medium. Volumetric yields were in the range of 14-16 g mannuronan per liter from 40 g fructose per liter medium, in approximately 35 hours fermentations. The *P. fluorescens* mutant Pf20118 derived from Pf201 was subject to more than 70 different experiments in fermentor, none of which have indicated instability in the mannuronan producing properties. Both Pf201 and Pf20118 have been grown for 60 generations without the appearance of non-mucoid colonies. Although it seemed probable that Pf20118 had a defect in the mannuronan C-5-epimerase gene *algG*, it could not be excluded that the mutations affected other proteins, which somehow could be necessary for epimerisation. A preliminary localization of the mutations responsible for the mannuronan-producing phenotype was performed by gene-replacement of the *algG* allele in each of the mutants by wild-type algG. A gene-replacement vector, pMG31, encoding wild-type *algG* and the first 135 bp of the downstream *algX* was constructed as described in Table 1. The plasmid was conjugated into the Pf20118 as described in Materials and Methods using PIA containing tetracycline as selective medium. Non-mucoid colonies appeared due to the disruption of the alginate biosynthetic operon as pMG31 recombined into *algG*. A non-mucoid transconjugant colony was incubated in 2-6 sequential liquid over-night cultures in the absence of tetracycline to allow loss of the integrated plasmid. Exponentially growing cultures were diluted 10^4-10^9 fold and

plated on PIA agar plates to screen for mucoid revertants. Mucoid colonies were then re-streaked on L-agar containing G-lyase to test if they produced epimerised alginate. Such non-mucoid revertants were found confirming that the mutation had to be in the DNA-fragment corresponding to the algGX' fragment of pMG31. The *algG*-gene was amplified by PCR using the primers PfalgG3r and PfalgG4f, sequenced and the mutation identified, confer Table 3.

Three other epimerase negative mutant derivative strains were prepared according to the procedure set forth above, and designated Pf2012, Pf2013, and Pf20137 respectively. They all have an identified mutation in their *algG* gene resulting in a different amino acid in their AlgG gene product , as set forth in Table 3 below, and this amino acid change is sufficient to inactivate the protein. The mutants yielded approximately the same levels of pure mannuronan as Pf20118, when grown under the same conditions. The epimerization defect of the mutants could be reverted by recombination with the wild type gene in pMG31.

Table 3 Mutations in *algG* in mannuronan-producing mutants

Mutant	Mutation in algG	Amino acid substitution in gene product
Pf2012	G(1081)→A(1081)	Asp(361) →Asn(361)
Pf2013	G(1289)→A(1289)	Gly(430) →Asp(430)
Pf20118	C(1222) →T(1222)	Arg(408) →Leu(408)
Pf20137	C(-3) →T(-3) C(1010) →T(1010)	- Ser(337) →Phe(337)

The mutant strains of table 3 were deposited in NCIMB under the accession numbers; Pf2012 has the NCIMB no. 41138, Pf2013 has the NCIMB no. 41139, Pf20118 has the NCIMB no. 41140 and Pf20137 has the NCIMB no. 41141.

Example 4

Preparation of acetylase negative and modified variant strains, Pf20118algFΔ and Pf20118algIJΔ.

A Pf20118 *algF* deletion mutant was first made by constructing a mutant DNA-fragment containing flanking sequences of an in-frame deletion of parts of *algF*, and then ligate the fragment into the suicide vector pMG48, as described in Table 1. The resulting plasmid, designated pMG79, was transferred to *P. fluorescens* strain

Pf20118 by conjugation as described in Materials and Methods, and the transconjugants were selected as blue colonies on PIA-plates containing XGal and tetracycline. Double recombinants were selected as white and mucoid colonies on PIA-plates containing XGal. These candidates were further tested for sensitivity to tetracycline. Twenty-four white, tetracycline sensitive candidates were tested by PCR using the primer-pair algF-1-fw and algF-2-Rev as given in Table 2, and the products were analyzed by gel electrophoresis. PCR-products from twenty-two of the candidates had the length expected for the wild-type *algF*-allele (1.0 kb). However, the two others had the expected length for the mutant Δ*algF*-allele (0.7 kb). One of these was designated Pf20118algFΔ.

A deletion mutant of *algIJ* was created by first creating a derivative (pMG49) of pMG27 from which a 1.4 kb *Nru*I-*Hpa*I DNA-fragment containing the 261 3' nucleotides of *algI* and the 5' 1140 nucleotides of *algJ* was removed. The deletion construct encodes an in-frame fusion of AlgI and AlgJ ensuring that AlgF and AlgA should be translated normally. A 3.4 kb *Sac*I-*Xba*I DNA-fragment from pMG49 was then ligated into the suicide vector pHE55 digested with the same enzymes, creating pMG50. This plasmid, containing the sequences flanking the deletion, was introduced to Pf20118 by conjugation from *E. coli* S17.1 and non-mucoid transconjugants were selected on PIA-medium with tetracycline. Transconjugant revertants were identified as mucoid tetracycline sensitive colonies on LA-medium. Four *algIJ*Δ-mutant candidates were tested by PCR-amplification of a region containing the deleted region using the primer pair PfacetylFw and PfacetylRev (Table 2) and the PCR-product was analyzed by agarose gel electrophoresis. Two of the colonies contained the wild type fragment (1.8 kb) while the two others contained the mutant segment (0.4 kb). One of these was designated strain Pf20118algIJΔ. Pf20118algFΔ and Pf20118algIJΔ were grown in fermentors using the PM5-medium and standard growth conditions as set forth in Materials and Methods, and the produced alginate was harvested and measured as earlier described. The results are given in Table 4 below. Both variants produced mannuronan alginate in yields of 16-17 g alginate per liter medium. The presence of acetyl groups was determined by ^{1}H-NMR-spectroscopy as described in Materials and Methods. Pf20118algFΔ did not produce acetylated alginate, while Pf20118algIJΔ produced alginate containing small amounts of O-acetyl groups.

Table 4 Alginate yield, fraction of guluronate residue content [F_G], degree of acetylation [da] and intrinsic viscosity [η] in fermentations with different *P. fluorescens* mutants

The fermentations were performed in 3-l fermenters using PM5-medium and standard growth conditions. Analyzes were done as described in Material and Methods.

Mutant	Alginate (g/l)	F_G (%)	da	η (dl/g)
Pf201	11,3	29	0,44	16,5
Pf20118	16,0	0	0,60	17,3
Pf20118 algIJΔ	16,8	0	0,03	10,9
Pf20118 algFΔ	16,2	0	0	8,9

Pf20118algFΔ and Pf20118algIJΔ are deposited in NCIMB under the accession numbers 41142 and 41143.

Example 5

Preparation of a modified derivative mutant strains displaying low alginate lyase activity, Pf20118AlgLH203R.

P. fluorescens has, according to current knowledge only one alginate lyase (AlgL) encoded by the gene *algL*. An option for controlling the molecular weight of the alginate produced by the bacterium, is therefore to modify the AlgL gene product simultaneously produced.

The mutagenic primer pair algLH203R1/algLH203R2 was used to create a His203Arg (H203R) mutation in the algL gene of Pf20118. The primers also contain silent mutations creating an *Age*I-site, for allele identification. The mutagenic plasmid pMG70 was constructed, as described in Table 1, and introduced to the Pf20118 chromosome by conjugation, and transconjugants were selected on PIA-medium with tetracycline and XGal. Transconjugants were grown in series of over-night cultures in the absence of tetracycline and plated on PIA-medium with XGal to isolate AlgL mutants. White tetracycline-sensitive mutant candidates were screened by PCR-amplification of the *algL*-allele using the primers PfalgL-BspHI-pMG26 and PfalgLRev1 (Table 2), and alleles were identified by digesting the PCR-fragment with *Age*I. The mutant strain chosen was designated Pf20118AlgLH203R.

When the variant strain Pf20118AlgLH203R is grown in shake flasks using the PIA-medium with reduced salt it yields amounts of mannuronan, approximately at the same level as the variant strain Pf20118. Also growth in fermentor led to approximately the same amounts of mannuronan produced from the two variant strains (H203R produced 12 g mannuronan alginate per liter). The intrinsic viscosity measurements of the mannuronan produced by Pf20118 (intrinsic viscosity of 15 dl/g) and Pf20118AlgLH203R (intrinsic viscosity of 37 dl/g) in shake flasks (using PIA medium with reduced salt, no proteases were added) show that the latter produces a mannuronan with increased molecular weight. Pf201 produced alginate with an intrinsic viscosity like Pf20118 (14 dl/g).

Bacterial cells of Pf201, Pf20118 and Pf20118AlgLH203R were harvested at the end of the fermentation in shake flask and sonicated. After sonication the extracts were investigated for alginate lyase activity, which was measured by the method as set out in Materials and Methods. Defining the lyase activity of Pf201 as 100 %, it is possible to detect activities down to 2 % using this method. Pf 20118 showed 93 % activity. No activity was detected for Pf20118AlgLH203R, indicating that it is less than 2 % of that of strain Pf201. Still, when the proteases Alkalase and Neutrase, both to 0.15 ml/l were added, the intrinsic viscosities increased to about 50 dl/g for Pf201 and Pf20118, and to 70 dl/g for Pf20118AlgLH203R, indicating that the mutant lyase has some residual activity. The variant strain Pf20118AlgLH203R is deposited in NCIMB under the accession number 41144.

Example 6

Preparation of variant mutant strains with reduced epimerase activity

The mutant strains Pf201 and Pf20118 provide the means to make alginate *in vivo*, with about 30 % guluronate residue content and pure mannuronan, respectively. Alginates with intermediate amounts of guluronic acid (guluronate residue) content that is between 0 and 30 % can however also be made.

A method for preparing variant strains with reduced epimerase activity is to exchange wild type *algG* with a mutant gene encoding a mutant protein with less activity. Four different amino acid substitutions are shown in Table 3 to give epimerase negative mutants of AlgG. In these four cases the amino acid change affect either the size or the charge of the amino acid, for two of them both properties

are changed. Alternative alleles of *algG* encoding more conservative changes in these amino acids is made by site specific mutagenesis using pMG26 as template. Mutagenic primers are made which contain a codon for the new amino acid flanked by about 10-15 nucleotides identical to the known sequence. Mutations in Ser337 will destroy the *Sma*I site, primers for the other amino acids do preferably contain silent mutations introducing a restriction enzyme site to aid in identifying the new mutant strains. Primers for both strands are to be synthesized, and the mutagenesis is performed as described in Material and methods. Mutated *algG*-alleles are then transferred to pMG48 digested with *Nsi*I-*Nco*I as a 2.7 kb *Bsp*HI-*Pst*I- digested DNA fragment. The resulting plasmids are transferred to Pf201 and transconjugants selected as being non-mucoid, tetracycline resistant, and blue on agar plates containing XGal. After growing selected clones for several successive transfers in LB-medium, double recombinants are selected as having white, mucoid colonies on agar plates containing XGal, and by being sensitive to tetracycline. *algG* from these candidates can be amplified using the primer pair PfalgG5f and PfalgG3r. The amplified product is 1.7 kb long. If a restriction site is removed, or introduced by the primers, the correct mutants are identified by using the corresponding restriction enzyme. Alternatively the candidates are confirmed by DNA-sequencing.

The mutant strains are grown in shaking flasks and the produced alginate is isolated as described in Materials and Methods. The amount of alginate and the G-content are determined using M- and G-lyases as described in Materials and Methods. The results from interesting strains are verified by NMR-spectroscopy.

Example 7

Preparation of variant mutant strain Pf201MC with an inducible *Pm* promoter for regulation of the alginate production

The *Pm* promoter together with its effector protein XylS is known to be a strong inducible promoter which can be used in many gram-negative species, Blatny et al., 1997, 63, Appl. Environ. Microbiol. p. 370-379. The inducer used is often toluate, which diffuses freely over the bacterial membranes. The *P. fluorescens* strain Pf0-1 has now been sequenced at JGI (http://spider.jgipsf.org/JGI_microbial/html/pseudomonas/pseudo_homepage.html). When the alginate operon of this strain was compared to known alginate operon

sequences from other *Pseudomonas* species, we found that the organization was similar. All sequenced alginate-producing species of *Pseudomonas* also have the same conserved open reading frame upstream of the alginate promoter. It potentially encodes a protein, the function of which is unknown. The objective of this experiment was to exchange the sequences downstream of the stop codon for this reading frame and upstream of the start codon of *algD*, the first gene in the alginate operon, with sequences encoding XylS, the Pm-promoter and the Shine-Dalgarno sequence from the vector pJB658 described in Blatny et al.,1997, 38, p. 35-51. Most of the DNA-segment separating *xylS* and the Pm-promoter in pJB658 was removed.

The first step was to clone the 3' part of the hypothetical protein (abbreviated *hyp*) and the 5' part of *alg*D in order to get flanking sequences for the insertion. When the sequences of *algEGXLIJFA* of strain Pf0-1 were compared to the sequences of NCIMB10525, it was found that the two sequences were not identical. The primers were therefore constructed using parts of the *hyp* and *algD* genes, which are highly conserved in several species. The 3' part of *hyp* was cloned as a 0.7 kb *Bsp*LU11I-*Spe*I-digested PCR fragment using the primers HypBspLU11I and HypSpeI of Table 2, into the suicide vector pMG48, generating pHE139. The 5' part of *algD* was cloned as a 0.8 kb *Nde*I-*Nsi*I restricted PCR-fragment into *Nde*I-*Pst*I-restricted pJB658celB, generating pHE138. The replacement vector pMC1, confer Figure 5 was then constructed through a series of cloning steps (Table 1).

The plasmid was transferred by conjugation to strain Pf201 as described in Materials and Methods, choosing XGal and tetracycline resistance/sensitivity to screen for recombinants and subsequent double recombinants. Colonies, which seemed to be more mucoid on PIA-medium containing 1 mM toluate, than on PIA-medium not containing toluate, were chosen for further analyses by PCR. Using the primer pair HypBspLU11I and AlgDNsiI (Table 2) the expected PCR-product from wild type strains would be 2.3 kb long, while that of the mutant strain would be 3.0 kb. The chosen mutant was designated Pf201MC. This strain was then fermented in the absence and presence of toluate (0.025 mM) as an inducer. PM5-medium and standard conditions were used during the fermentation. The un-induced culture produced 3.5 g alginate per liter, whereas the induced culture produced 13 g alginate per liter. The mutant strain Pf 201MC is deposited in NCIMB under the accession number 41145.

Example 8

In vitro epimerization of mannuronan alginate product

Mannuronan produced as described in example 4, was dissolved in buffer (Mops (50 mM), $CaCl_2$ (2,5 mM), NaCl (10 mM), pH 6,9) to a concentration of 0,25 % mannuronan alginate. The mannuronan C5-epimerase AlgE4 produced and purified as described by Ramstad et al, Enzyme and Microbial Technology, (1999), 24, p 636-646, was added to a concentration of 1 mg enzyme / 200 mg mannuronan. The solution was incubated at 37°C for 23 hours. The epimerization was stopped by acid precipitation of the alginate. The alginate was then redissolved in distilled water and neutralized with alkali. The alginate solution was added NaCl to a concentration of 0,2% and one volume of ethanol (96%) to precipitate the alginate. The precipitated alginate was washed 3 times with 70% ethanol, and 2 times with 96% ethanol, and freeze dried. The freeze-dried alginate was treated further and analyzed by NMR as described in Materials and Methods. The product after this incubation was an almost totally polyalternating alginate (PolyMG,Table 5).

Poly MG was redissolved in buffer (Mops (50 mM), $CaCl_2$ (2,5 mM), NaCl (10 mM), pH 6,9). The mannuronan C5-epimerase AlgE1 was produced and purified as described by Ramstad et al, Enzyme and Microbial Technology, (1999), 24, p 636-646, except that it was purified by ion-exchange chromatography only. It was added to a concentration of 1 mg enzyme / 200 mg mannuronan. The solution was incubated at 37°C for 4 days. Additional AlgE1 (1 mg enzyme / 200 mg mannuronan) was added after 1, 2 and 3 days incubation. The epimerization was stopped as described above. Alginate was isolated and analyzed by NMR as described above. The result of the epimerization was an alginate with a G-content of >95 % (Table 5).

Table 5. Composition of alginate after epimerization of mannuronan with AlgE4 and AlgE1

Alginate	FG	FM	FGG	FGM/MG	FMM
Mannuronan	0	1,0	0	0	1,0
PolyMG (mannuronan + AlgE4)	0,45	0,55	0	0,45	0,1
Poly G (PolyMG + AlgE1)	>0,95	<0,05	>0,9	<0,05	<0,02

Patent claims

1. Biologically pure bacterial culture of at least one mutant strain of *P. fluorescens*, wherein said strain produces at least 10 g alginate per liter medium.

2. Biologically pure bacterial culture of at least one mutant strain of *P. fluorescens*, wherein said strain produces at least 10 g alginate per 40 g carbon source per liter medium.

3. Pure mutant strain of *P. fluorescens* of claim 1 or 2, wherein said mutant strain is selected from the group consisting of the mutant strain Pf201, Pf2012, Pf2013, Pf20118, Pf20137, Pf20118algIJΔ, Pf 20118algFΔ, Pf20118AlgLH203R and Pf201MC.

4. Pure mutant strain of *P. fluorescens* of the claims 1, 2 or 3, wherein the said mutant is capable of producing large amounts of an alginate consisting of mannuronate residues only.

5. Pure mutant strain of *P. fluorescens* of the claim 4, wherein the said mutant is selected from the group consisting of the variant strains Pf2012, Pf2013, Pf20118, and Pf20137.

6. Pure mutant strain of *P. fluorescens* of claim 1 or 2, wherein the said mutant is capable of producing alginate having a defined guluronate residue (G)-content between 0 and 30 %.

7. Pure mutant strain of *P. fluorescens* of the claims 1, 2 or 3, wherein the said mutant is capable of producing alginate without, or with a reduced number of O-acetyl groups.

8. Pure mutant strain of *P. fluorescens* of the claim 7, wherein the said mutant is selected from the group consisting of the mutant variant strains Pf20118algIJΔ and Pf 20118algFΔ.

9. Pure mutant strain of *P. fluorescens* of claim 1, 2 or 3, wherein the said mutant is capable of producing alginate with a molecular weight of between 100,000 and 3,000,000 Daltons.

10. Pure mutant strain of *P. fluorescens* of claim 9, wherein the said mutant is selected from the group of the variant mutant strain Pf20118AlgLH203R.

11. Pure mutant strain of *P. fluorescens* of claim 1, 2 or 3, comprising an alginate biosynthetic operon regulated by an inducible promoter different from the naturally occurring promoter, and optionally one or more effector genes.

12. Pure mutant strain of P. fluorescens of claim 11, wherein the inducible promoter is a *Pm* promoter, and further comprising the effector gene *xylS*.

13. Pure mutant strain of *P. fluorescens* of claim 11 and 12, wherein the said mutant strain is Pf201MC.

14. Method of producing the novel mutant strain of *P. fluorescens* of claim 1 or 2, wherein :

(a) a wild-type strain of *P. fluorescens* is contacted with a mutagenic agent, and
(b) the treated bacteria of step (a) are grown in the presence of one or more antibiotics, and
(c) antibiotic resistant mucoid mutants are isolated by selection, and
(d) the alginate production properties of the isolated mucoid mutants of step (c) are determined.

15. Method according to claim 14, wherein the mutagenic agent is nitrosoguanidine.

16. Method according to the claims 14 or 15, wherein the treated bacteria of step (a) are grown in the presence of a β-lactam or aminoglycoside antibiotic.

17. Method according to claims 14 or 15 wherein the treated bacteria of step (a) are grown in the presence of carbenicillin.

18. Method of producing a mutant strain of *P. fluorescens* of claim 11, wherein
(i) the alginate biosynthetic operon promoter of a wild type strain of *P. fluorescens* is exchanged by an inducible promoter by homologous recombination, and
(ii) optional effector genes are introduced into the bacterium of (i) by homologous recombination, transposon mutagenesis or by means of a plasmid, and
(iii) mutants are grown and then isolated by selection, and
(iv) the alginate production properties of the isolated mutants of (iii) are determined.

19. Method according to claim 18, wherein the inducible promoter is *Pm* from *P. putida* Tol-plasmid.

20. Use of biologically pure bacterial culture of at least one mutant strain of *P. fluorescens* of any of the previous claims 1-13, for the production of alginate.

21. Use of the alginate produced by at least one mutant strain of *P. fluorescens* of any of the previous claims 1-13, in the preparation of a pharmaceutical or nutrient product, or as an intermediate product for *in vitro* C-5-epimerization.

Abstract

It is described a biologically pure bacterial culture of at least one mutant strain of *P. fluorescens*, which produces large amounts of alginate. The alginate may contain a certain determined content of mannuronate and guluronate residues, possible presence and determined level of acetyl groups in the alginate, and a desired molecular weight of the alginate. Also high yielding mutants with regulation of alginate production, is described. The invention further provides methods for producing new mutant strains of *Pseudomonas fluorescens* and variants thereof, and use of the resulting strains in alginate production.

Figure 1

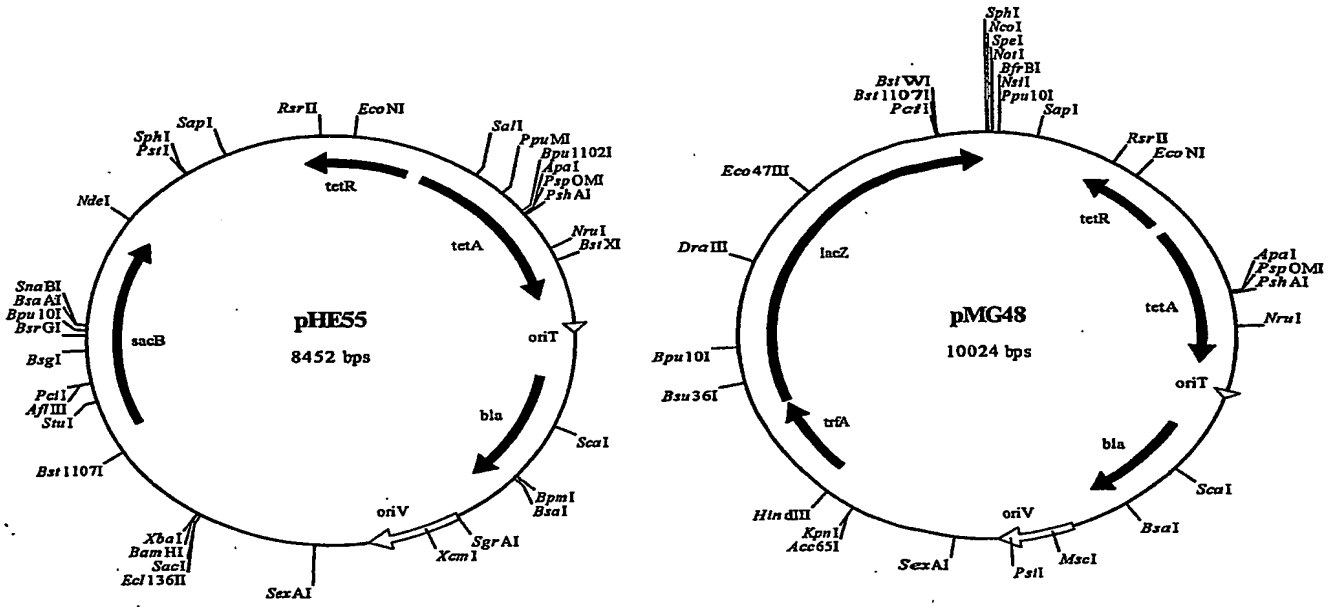

Figure 1

Restriction map of the suicide plasmids pHE55 and pMG48.

Figure 2

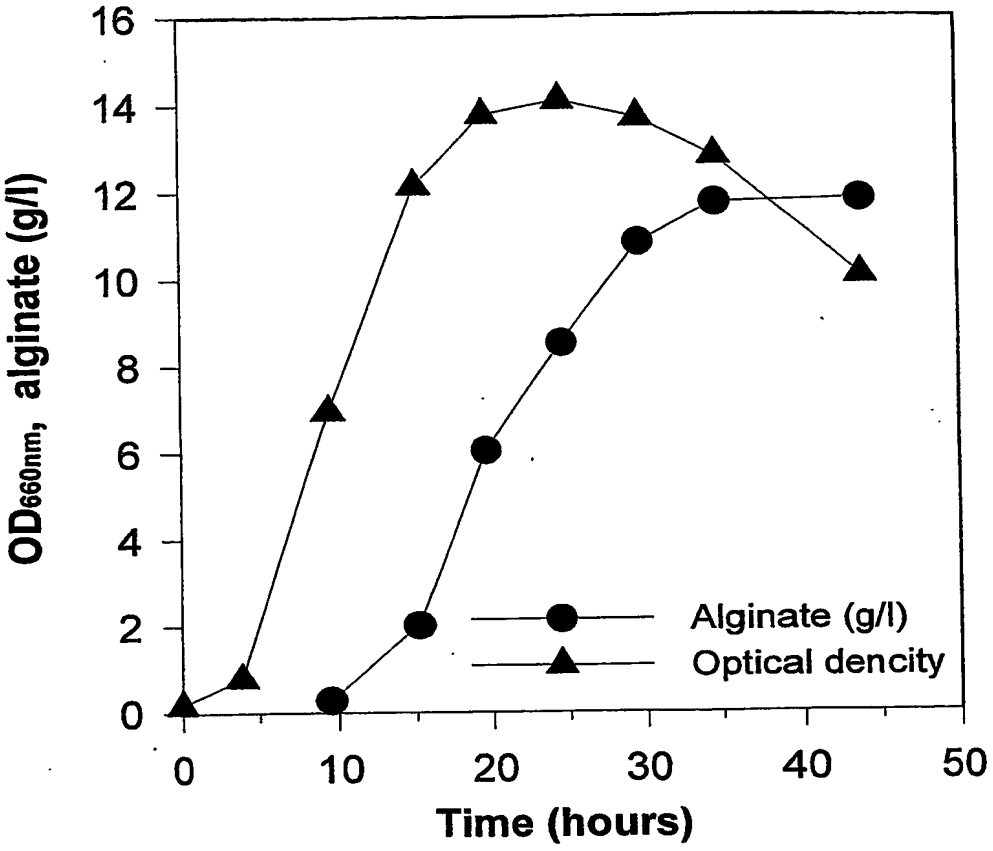

Figure 2. Growth and alginate production in fermentation with strain Pf201.

Figure 3

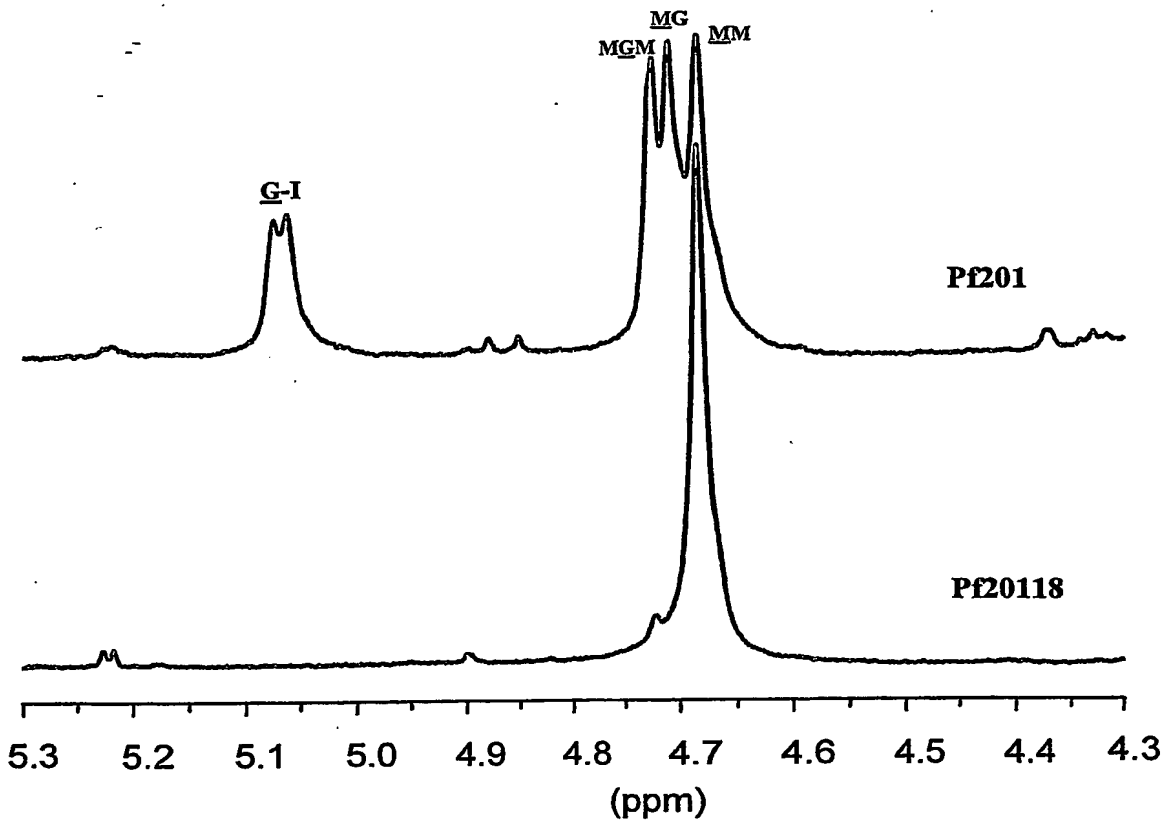

Figure 3. ^{1}HNMR-spectra of alginate produced by P. fluorescens 201 and mannuronan produced by *P. fluorescens* 20118

Figure 4

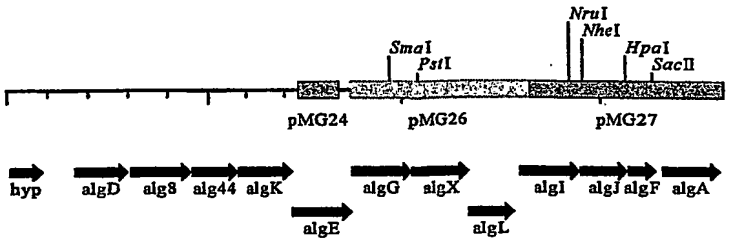

Figure 4: The alginate biosynthetic operon and the upstream open reading frame from *P. fluorescens*. The cloned fragments are marked as boxes on the mapline.

Figure 5

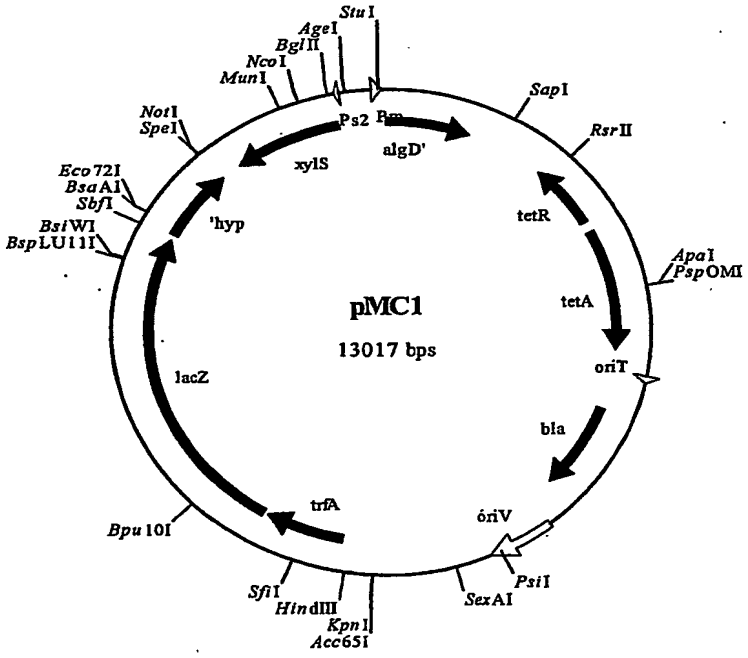

Figure 5: Restriction map of pMC1.